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The identification of single strand DNA aptamers which specifically bind to platelets using cell-SELEX technique

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ABSTRACT

Aptamers are oligonucleotides that can be easily synthesized and bind to their targets with high affinity and specificity. Several aptamers specific to soluble factors of coagulation cascade have been produced, however, aptamers specific to platelet cell membrane molecules have not been reported yet. We aimed to discover DNA aptamers that specifically bind to human platelets. The cell-SELEX method was used for aptamer discovery. Synthetic 79 nucleotides length single-strand oligonucleotides were used as a library. Ultra-pure platelets were prepared using differential centrifugation steps and magnetic-bead-assisted removal of contaminating cells. The FITC-labeled forward primer was used for amplification of the selected oligonucleotides by PCR, and Lambda exonuclease was used for digestion of the lagging strand. After 12 rounds of cell-SELEX, selected oligos were amplified and cloned to pTG19-T vector, transfected into *E. coli* (TOP10) and sequenced. Sequences of aptamers from 200 individual positive colonies were aligned and seven clusters were identified. Representative aptamers were amplified and their affinity, specificity, and digestibility of their targets were evaluated. Interferences of the aptamers to two platelet function tests were also investigated. Affinity (K_D) of the representative aptamers were between 109 and 340 nM. Trypsin exposure of the platelets completely abolished the binding of the 7 aptamers to the targets. The binding of the four aptamers fully protected their target molecules from digestion. No one of the aptamers changed the parameters of the platelet function tests. Seven aptamers specific to platelets were identified and characterized. These aptamers may have potentially diverse applications in the diagnosis or treatment of platelet disorders.

Keywords

Cell-SELEX; platelet; DNA aptamer; Platelet-specific aptamer.

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Abbreviations

SELEX: Systematic evolution of ligands by exponential enrichment

K_D: Dissociation constant
PCR: Polymerase chain reaction
SEM: Standard error of the mean

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Introduction

Nucleic acid aptamers are promising alternatives to antibodies in life science and because of their unique properties, have gained increasing attention from scientists. They are small (25-100 bp), single-stranded, synthetic nucleic acids [1] which can be folded into their 3D conformation capable of binding to certain targets with extremely high affinity and specificity. They can be easily synthesized, modified, or conjugated by chemical reactions. The aptamer can be denatured and renatured frequently without loss of its activity. They are more than 15-20 times smaller than antibodies, therefore, can easily diffuse across cell membranes or penetrate areas that are not accessible for antibodies [2]. Aptamers also possess little or no immunogenicity and low systemic toxicity in vivo [3].

Systematic evolution of ligands by exponential enrichment (SELEX) technology for selection and enrichment of aptamers has been explained in detail elsewhere [4,5]. Briefly, a single-stranded nucleic acid pool is prepared and incubated with the target molecules, to recover oligonucleotide variants with desired binding affinity to the targets. Bound oligonucleotides are isolated from their targets and amplified using conventional polymerase chain reaction (PCR). The selection and amplification cycles are sequentially repeated and the ligand-specific signal intensity is determined after each round of selection until the gradual increases in the signal intensity reach the plateau state (no further increase in the signal intensity between two or three successive rounds) [5].

Initially, aptamers have been produced solely against small molecules in a pure state [6]. Therefore, production of aptamers specific to cell membrane-associated molecules had been feasible only when the molecules had been prepared in the pure form, however, the purification process is usually associated with denaturing and disturbing the native conformation of the molecule, and aptamers produced using a denatured form of the molecule, probably, will not be able to recognize and bind to their targets that are in native forms [5,7].

The first complex matrix that was used as a target for aptamer discovery was bacterial ribosomes [8]. Morris et al produced high-affinity aptamers against red blood cells in 1998 [9]. Since then, many efforts have been made to develop high-affinity aptamers against eukaryotic or prokaryotic cells [10]. Cell-SELEX, as performing SELEX using a homogeneous population of eukaryotic or prokaryotic cells, has been introduced for the simultaneous development of numerous aptamers against cell-associated molecules. Recently, Cell-SELEX technology has been increasingly used in biomarker discovery [11,12]. Prior knowledge of the target molecule is not required for the development of a target-specific aptamer through cell-SELEX. The cell membrane surface has a countless number of molecules, therefore at the end of a successful cell-SELEX procedure, numerous aptamers are generated for many different targets.

Aptamers have been used as drug delivery agents with tunable release capacity or antidote-assisted prevention of drug-related side-effects [13]. They are also used for biomarker discovery or purification of target molecules [14,15]. They have found also applications in diagnostic approaches, e.g., ELISA [16,17], or biosensor design [18]. Aptamer attachment may also inhibit the biological activities of the target molecule or may prevent it from enzymatic digestion [19,20].

Platelets are small, anucleate, disc-shaped cells in blood with dimensions ranging 2-4 micrometers. They actively contribute to blood hemostasis, inflammation, host defense, tumor growth, and metastasis. After red blood cells, platelets are the second abundant cells in blood with normal platelet counts ranging from 1.5-4.5 x 10^5 cells per microliter. Any abnormalities in platelets can lead to various bleeding disorders [21]. Platelets have receptors and adhesion molecules on their surface to interact with immune cells and also with circulating pathogens [22]. Blood platelets are usually brought into close contact and have frequent interactions with vascular walls, red blood cells, leukocytes, other platelets, plasma contents, and even with foreign bodies penetrated the circulation (e.g., infectious agents or toxins). Platelet membrane-associated molecules play a major role in the reciprocal interactions. Several aptamers have been produced specifically to soluble factors of the coagulation cascade [23], however, to the best of our knowledge, aptamers specific to platelet membrane molecules have not been produced yet. Our goal was to produce DNA aptamers that bind specifically to platelet membrane-associated molecules.

Abbreviations-Cont'd

LTA: Light transmission aggregometry
PFA-100: Platelet function assay 100
MAR: Maximal aggregation rate
WBC: White blood cell
RBC: Red blood cell
PGE1: Prostaglandin E1
ASA: Acetylsalicylic acid, Aspirin
P2Y12: An ADP receptor
COX: Cyclooxygenase
PT-VWD: Platelet type von Willebrand factor
GPI: Glycoprotein I
ADP: Adenosine diphosphate
EDTA: Ethylene diamine tetra acetic acid
ACD: Acid citrate dextrose
PRP: Platelet rich plasma
PPP: Platelet poor plasma
RT: Room temperature
LPA: Linear polyacrylamide
CEPI: Collagen-Epinephrin
CAPD: Collagen-ADP

Results

Quality and purity of cells

Through the cell preparation step, platelets of 3x10 ml blood samples (per day) from three fixed donors(O+) were concentrated in one ml. Our mission was to obtain highly pure target cells to apply in the aptamer discovery process and all conditions were adjusted to achieve the goal. Magnetic beads equipped with RBC- and WBC-specific antibodies swept all unwanted cells from the suspension, including activated platelets complexed with the cells. During the 13 working days (2-4 weeks intervals), a total of 39 blood samples were drawn and platelet density was measured in the initial samples and the final concentrates. Platelet recovery rates were ranged between 62-68% (mean ± SEM: 65.69 ± 0.56 %). Inclusion of the inhibitors in the anticoagulant solution efficiently prevented platelet activation during the purification process confirmed by flow cytometric analysis of the activation and apoptotic markers in the collected cells using anti-CD62p-PE and Annexin-V-FITC (figure 1). Concomitantly, a highly pure suspension of white/red blood cells was prepared for counter selection. The viability of the cells assayed by the Trypan blue exclusion method was 0-2%.

Visualization of the PCR product: Rapid and effective way for a demonstration of the efficacy of the PCR reagents and the entire process is a visualization of the products using agarose gel electrophoresis. PCR amplified samples from eight rounds of SELEX were resolved on 3% agarose gel with TBE (0.5x) as running buffer and DNA safe-stain as coloring agent (Figure 2B).

Single-strand preparation: Oligonucleotides collected from each round of SELEX were amplified through PCR assay using FITC-labeled sense primer and 5'phosphorylated antisense primer. For the next round of SELEX single-stranded FITC-labeled oligonucleotides are required, though, the phosphorylated strand was digested using Lambda exonuclease enzyme. For demonstrating the reliability of the enzyme and for optimizing reaction conditions, double-strand PCR products were exposed to the enzyme for 0, 10, 20, 30, and 40 minutes at 37 ℃ obeying the supplier's instructions. Agarose gel electrophoresis (3% and 10%) for resolving the single-strand preparation was not successful and was associated with a fully smeared electrophoretic band (data not shown). We took the advantage of native polyacrylamide gel (10%, TBE as running buffer, and Methylene blue as coloring agent) electrophoresis for visualization of the rescued strand (figure 2A).

Monitoring the efficiency of enrichment process

Totally 13 rounds of SELEX were applied and ssDNA samples were collected for flow cytometric evaluation of the enrichment process. Figure 3 shows the efficiency of the enrichment process during the progression of the SELEX toward the 13th round.

Characterization of the selected aptamers: Plasmids originating from the 200 positive colonies were

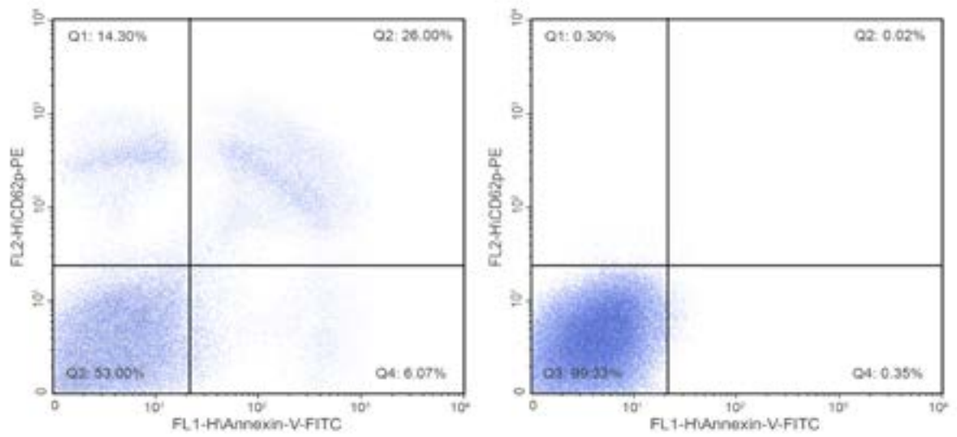

Figure 1.
Evaluation of platelet activation and apoptosis markers in highly purified platelets. A. Dot plot diagram of collagen-treated platelets (as a positive control) showing high frequencies of activated and apoptotic cells stained by anti-CD62p-PE and Annexin-V-FITC. B. Dot plot diagram of purified platelets showing very low frequencies of cells stained by the two fluorochromes. Quadrants were drawn using isotype controls.

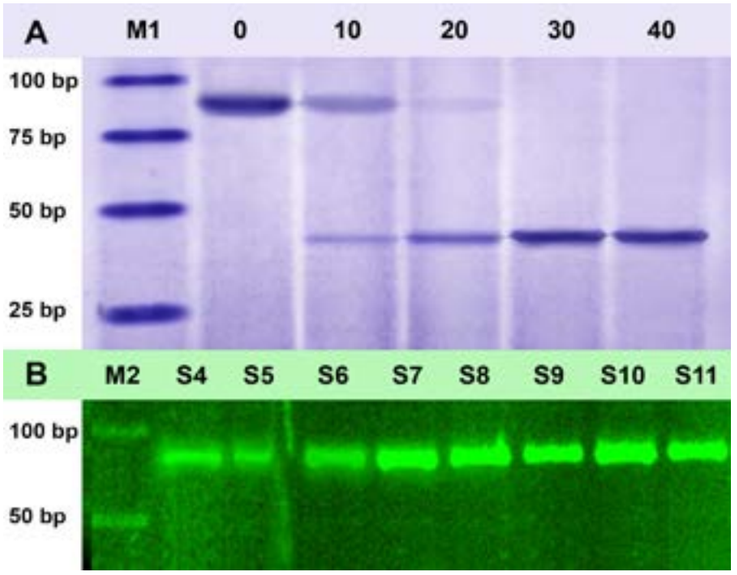

Figure 2.
Results of Native-PAGE and Agarose gel electrophoresis. A) native polyacrylamide gel electrophoresis of double-strand PCR products exposed to Lambda exonuclease enzyme for different time points (0, 10, 20, 30 and 40 minutes). M1: 25 bp DNA Ladder. Digestion of phosphorylated strands of double-strand PCR products was complete after 30 minutes exposure to the enzyme. B) Agarose gel electrophoresis of PCR products from 8 rounds of SELEX. Oligonucleotides collected from each round of SELEX were amplified by PCR. Five µL from the PCR product was mixed with 1 µl loading buffer and resolved by 3% agarose gel electrophoresis (100V). DNA safe stain was used for visualization. M2: 50 bp DNA ladder. S: SELEX.

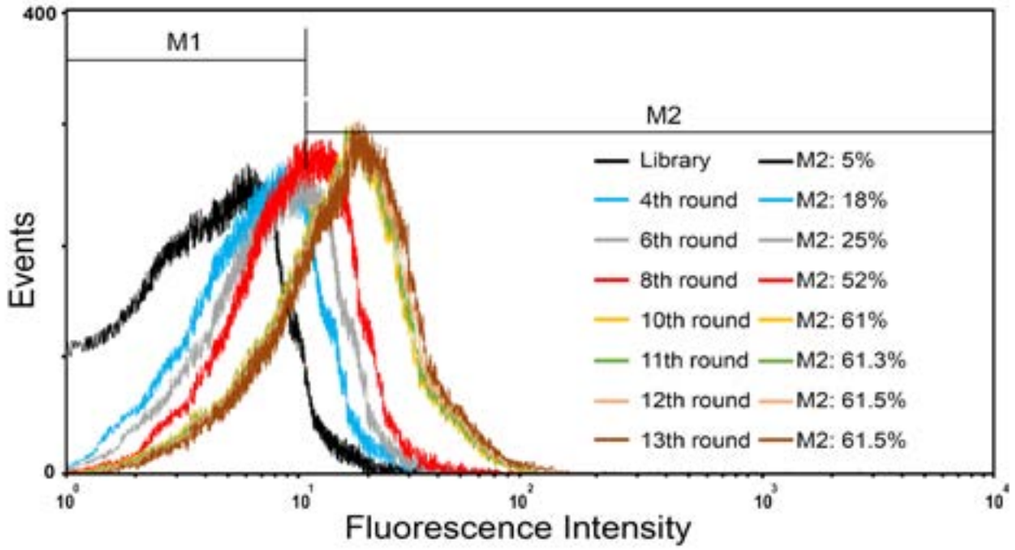

Figure 3.
Frequency of positive cells stained with FITC-labeled aptamers. PCR products from eight rounds of SELEX were incubated with fixed numbers of pure platelets and analyzed. M1 and M2 regions were drawn using control library and adjusted to contain 95% and 5% of the cells, respectively. The same regions were used for segregation of the positive cells stained by outputs of the later rounds. As the histograms show, gradual increases in the frequency of positive cells were observed during the SELEX progression and the frequency reached from M2: 5% in the first round to M2: 61.5% in the 12th round and not changed during the 13th round.

sequenced, aptamer sequences aligned and divided into 7 clusters (Table 1) based on the sequence similarity. Sequence variation within each cluster was presented in figure 4. 2D structure of the seven representative aptamers was presented in Figure 5. Figure 6 shows the binding affinity and calculated K_D values of the aptamers.

Effects of Aptamers on platelet function in vitro

Seven aptamers at three concentrations were incubated with platelets and subjected to aggregometry by the LTA method in the presence of collagen or epinephrine. Slop and MAR was determined and compared (figure 9A/9B). No one of the seven aptamers had noticeable effects on slope or MAR of the platelets by the LTA method (paired-samples *t*-test: $p > 0.05$). PFA-100 test results were also not significant compared to the controls (figure 9C/9D).

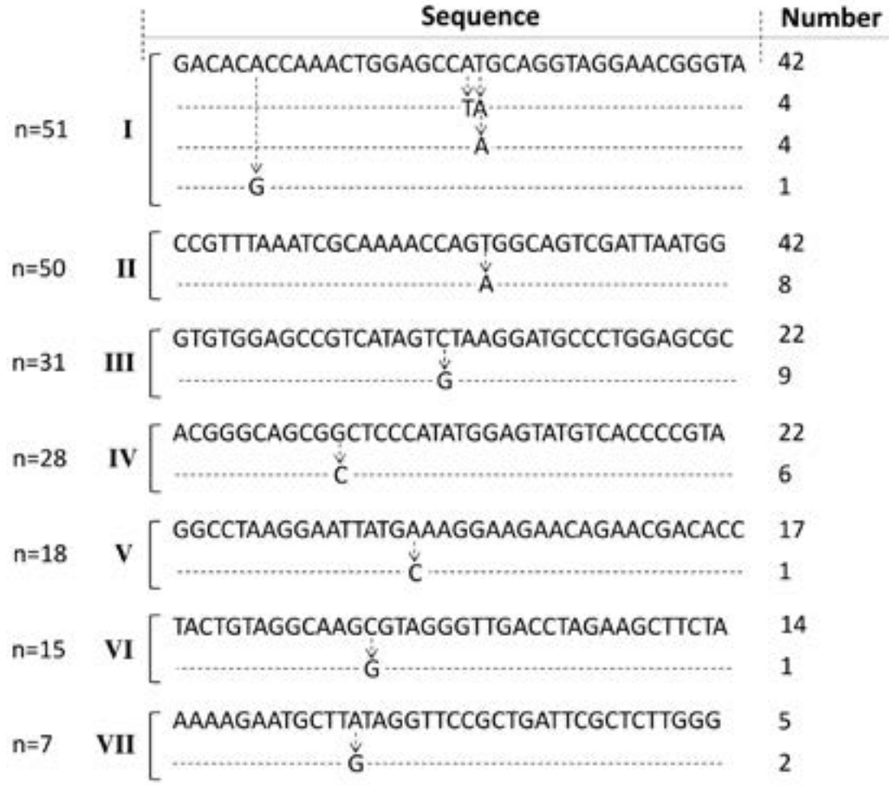

Figure 4.
Nucleotide variation within each cluster. Total number of oligos in each cluster was presented in the left. Number of sequenced oligos in each variant was provided in the right. Roman numerals (I-VII) show cluster numbers.

Table 1.
Nucleotide sequences of the seven aptamers (without fixed regions).

No.	Name	Sequence
1	APT1	GACACACCAAACTGGAGCCATGCAGGTAGGAACGGGTA
2	APT2	CCGTTTAAATCGCAAAACCAGTGGCAGTCGATTAATGG
3	APT3	GTGTGGAGCCGTCATAGTCTAAGGATGCCCTGGAGCGC
4	APT4	ACGGGCAGCGGCTCCCATATGGAGTATGTCACCCCGTA
5	APT5	GGCCTAAGGAATTATGAAAGGAAGAACAGAACGACACC
6	APT6	TACTGTAGGCAAGCGTAGGGTTGACCTAGAAGCTTCTA
7	APT7	AAAAGAATGCTTATAGGTTCCGCTGATTCGCTCTTGGG

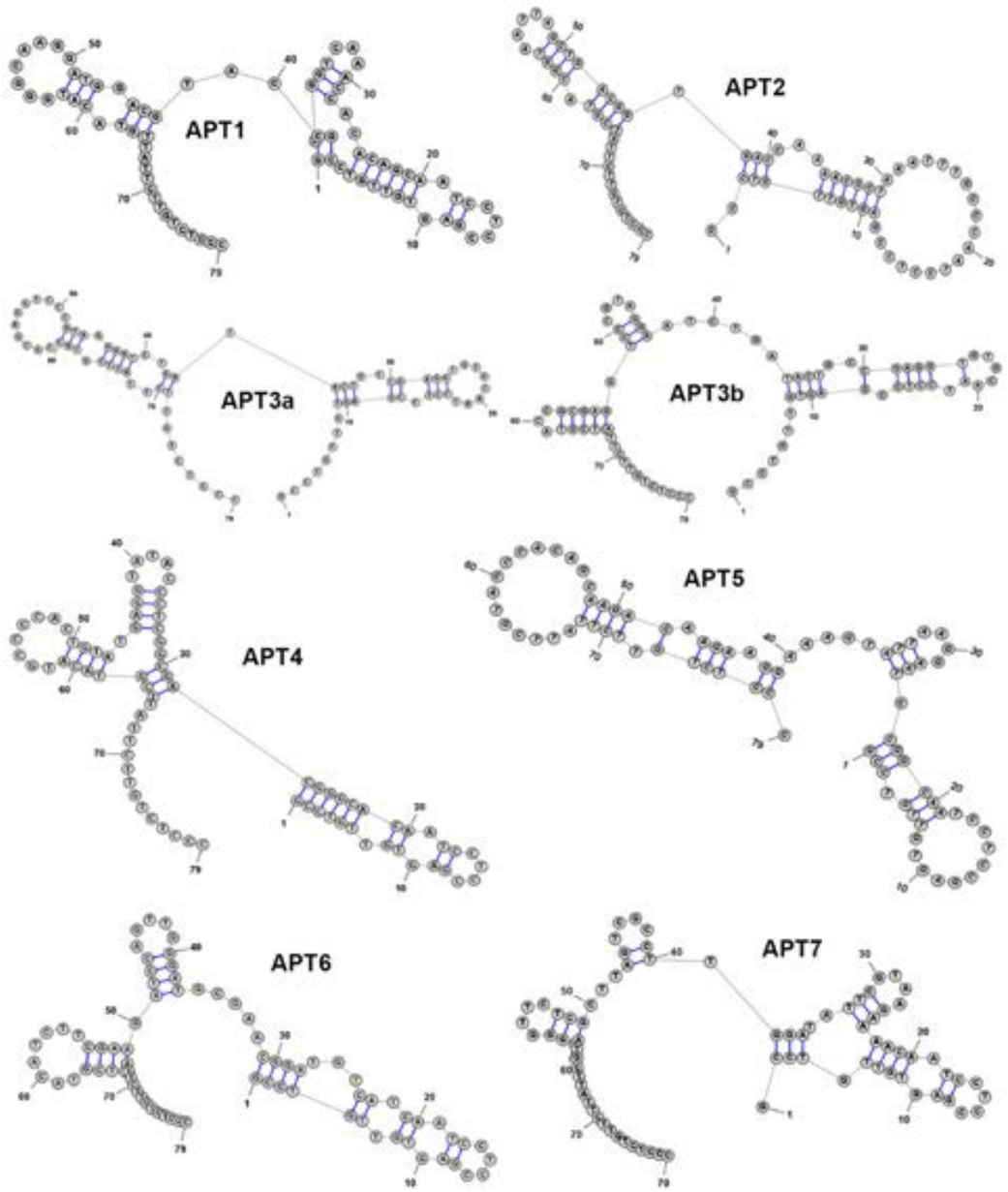

Figure 5.
Configurations of the representative aptamers (graphed by RNAstructure 6.2 software). Ionic strength was set to the physiological conditions (144 mM Na^+) and temperature was set to 21 °C.

Discussion

Our goal through the current study was the discovery of high affinity and specificity DNA aptamers against human platelets and the cell-SELEX method introduced by Sefah [5] was used for the experimental process. Ultra-highly pure and intact cells were needed for a successful cell-SELEX procedure; however, platelets are very susceptible to physical and chemical stimuli and are highly prone to activation. In the circulation, platelets are prohibited from unnecessary activation, however, upon draining, platelets come into close contact with foreign surfaces and are fairly activated. Upon activation, cell structure and morphology undergo tremendous changes and the cells adhere to other cells and surfaces leading to the cell aggregation and release of a huge number of chemicals. Therefore, the preparation of highly pure intact platelets requires the implementation of optimized protocols and unique protecting materials. The Amisten [24] method with some modifications was selected and used for cell purification. The method had been optimized by Amisten for mRNA detection by microarray analysis and had four main phases: blood collection, PRP preparation, leukocyte removal by filtration, and magnetic bead-assisted depletion of contaminating cells. We modified the method for achieving the highest platelet recovery rate and purity.

The highest platelet recovery rate had been reported by Wrzyszcz [25] which was 53.3 ± 13.1 (%), while the recovery rate in the current study was estimated to be 65.69 ± 0.56 (%). The first step in PRP preparation is the precipitation of the red and white blood cells by a low-speed centrifugation step, while, a substan-

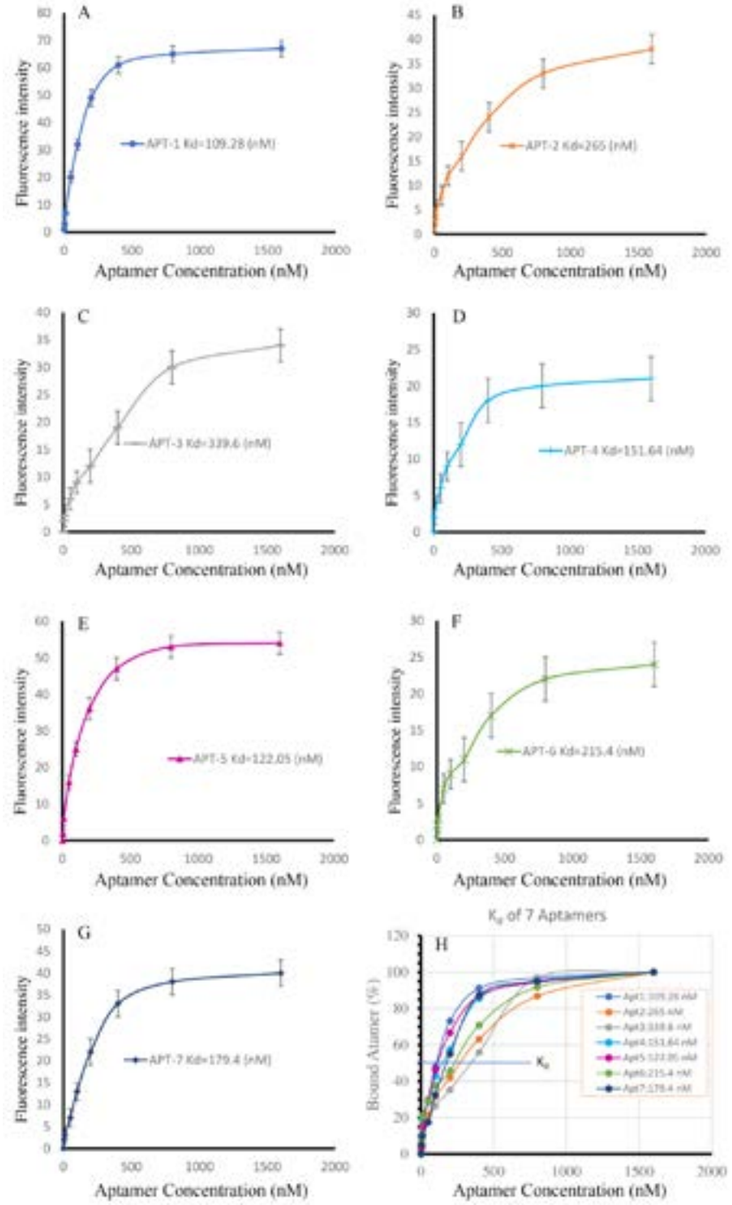

Figure 6.
Binding affinity of seven aptamers to platelets and their K_D values. A to G presented geometric mean fluorescence intensity of each concentration of the aptamer that bound to fixed number of platelets in vertical axis. Error bars presented SD values calculated using triplicate experiments. The highest levels of fluorescence (plateau tail of the curve) are different between aptamers, probably, due to the different densities of the target molecules on the platelet surface. H shows K_D values in a comparable manner between aptamers. Vertical axis presents fluorescence intensity of each experiment relative to the maximum fluorescence from the same aptamer, expressed as percent. K_D values were estimated using one-site saturation equation (see text).

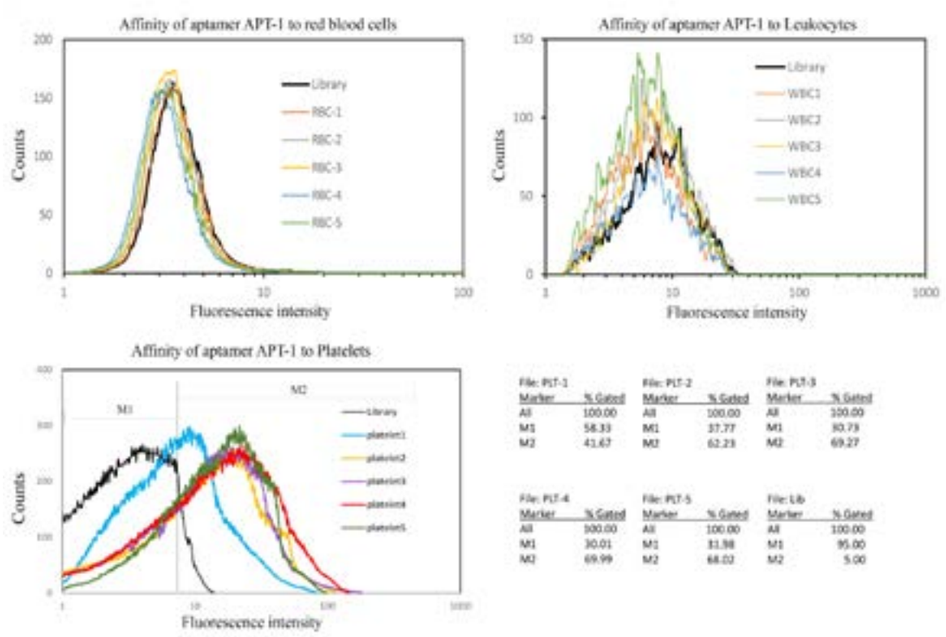

Figure 7.
Affinity of aptamer APT-1 to pure cells from five donors. For selectivity assessment pure RBC, WBC and platelets from five donors were incubated with aptamer APT-1 and fluorescence signals from cell-bound aptamers were analyzed by flow cytometry. Bold black line represents control library. Fluorescence intensity of APT-1 aptamer bound to RBCs or WBCs (five color lines) were exactly equal to the control library, therefore, APT-1 aptamer to not have any affinity to the cells. In contrast, platelets from the five donors all captured APT-1 aptamers and resultant fluorescence signal intensities were obviously higher than control library. M2 regions for platelet1-5 contain 41.67%, 62.23%, 69.27%, 69.99%, 68.02% of cells while the initial library was set to contain only 5% of cells in the region.

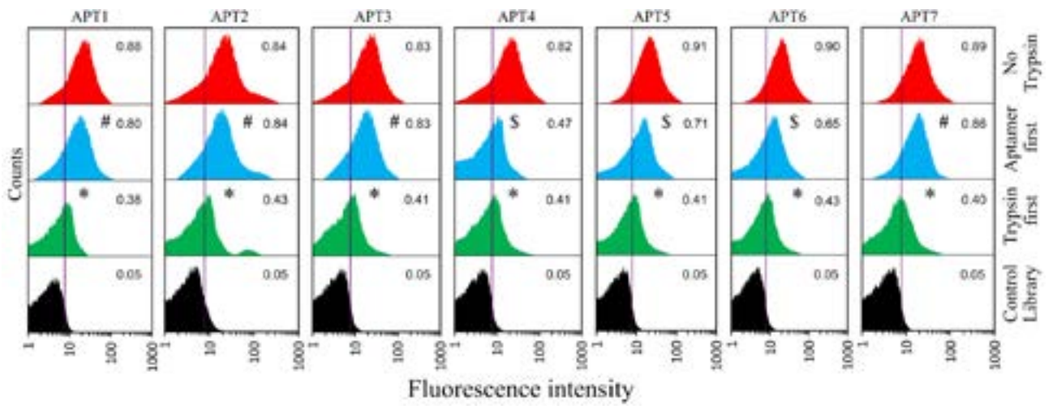

Figure 8.
Digestibility of ligands before and after aptamer binding. Platelets were treated with trypsin before (green histograms) or after (blue histograms) incubation with aptamers APT1-APT7. Black histograms represent control library (5% positive). Red histograms show platelets not treated with trypsin but exposed to the seven aptamers. Asterisk signs (*) mark all green histograms displaying a significant reduction in the fluorescence intensity (shift to the left compared to the red histograms) that means ligands have been digested by the enzyme activity. Hash signs (#) mean that aptamer binding completely protected ligand against trypsin digestion. Dollar signs ($) means partial protection of ligand after aptamer binding.

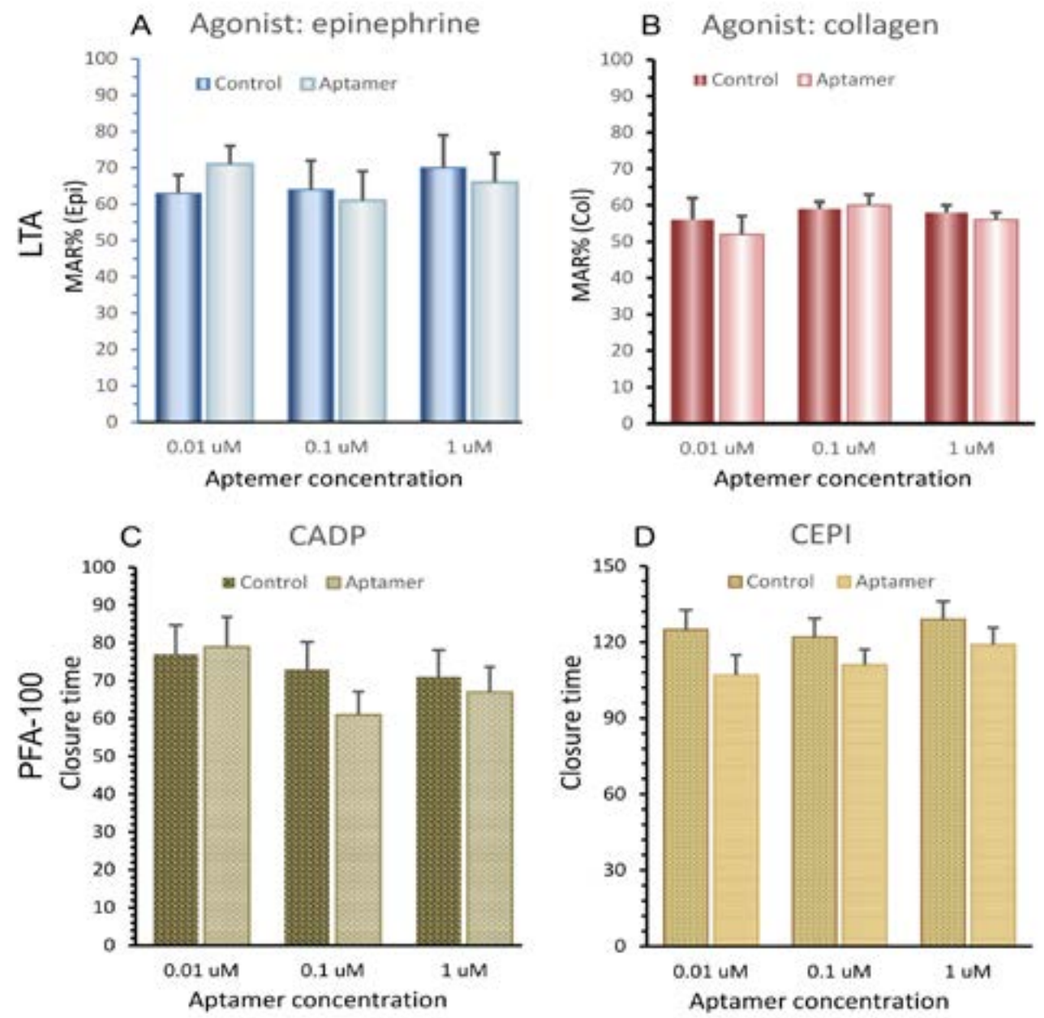

Figure 9.
Aptamer effects on two conventional platelet function tests: LTA and PFA-100. A&B: Aptamer effects on the results of light transmission aggregometry (LTA). C&D: Aptamer effects on the platelet function assay with PFA-100 instrument. Three concentrations (0.01, 0.1, 1 µM) of aptamers were prepared using routine samples (platelet-rich plasma in LTA assay and citrated blood in PFA-100 test) and assayed. Control samples prepared using initial library (instead of select aptamer). Epinephrine (A) or Collagen (B) were used as agonists in LTA assay. Cartridge in the PFA-100 tests equipped with Collagen + ADP (C) or Collagen + Epinephrine (D). Results of LTA assay expressed as mean "maximal aggregation rate" (MAR: %) and results of PFA-100 test expressed as mean "closure time" (second). All seven aptamers were analyzed, however, results of APT1 only presented here. Paired samples t-test was used for statistical comparisons. In collection, probably, due to the high variability of the data obtained from these tests, statistical comparisons did not demonstrate any significant differences between groups. Calculated coefficient of variations (CV) for LTA (COL/EPI) and PFA-100 (CADP/CEPI) assays were 15%/18% and 6.5%/7.9%, respectively.

tial amount of the platelets is also coprecipitated along with the cells. Platelet poor plasma, PPP, was prepared and added to the precipitated cells and gently mixed by upside-down method, and centrifuged again for the recovery of the coprecipitated platelets.

A filtration step has been proposed by Amisten to remove WBCs, however, platelet activation was seen during the process, and subsequently, the filtration step was omitted and the entire process was optimized to achieve the highest purity and intactness. In addition, leukocyte removal filters did not remove erythrocytes, a drawback that has also been reported

by Wrzyszcz [25].

Platelet activation is associated with a major change of the molecular composition of the cell membrane and triggers platelet adherence to the adjacent cells and surfaces. For achieving the highest cell recovery rate and keeping intactness of the cells, PGE1 and ASA were added to the anticoagulant solution and working buffers. ASA covalently binds to Ser530 in the cyclooxygenase, COX, molecule and hinders the access of arachidonic acid to the catalytic site of the enzyme; therefore, prohibiting thromboxane synthesis. Prevention of thromboxane generation by ASA prevents fibrinogen receptor activation and impairs platelet-platelet interactions [26]. PGE1 virtually antagonizes P2Y1 receptor activation on the platelet membrane. PGE1 binds to its receptor on the platelet and inhibits phospholipase C enzyme activation through increased production of cAMP that eventually inhibits mobilization of intracellular calcium and cell aggregation [27].

Through the cell preparation step, experimental conditions for differential centrifugation were optimized and magnetic bead-assisted removal of the contaminating cells was applied. Magnetic beads equipped with anti-CD45 (a common leukocyte marker) or anti-CD235a (a common erythrocyte antigen) were used for further purification of platelets and in the same manner, magnetic beads equipped with anti-CD41 (a common platelet marker) was used for further purification of WBC and RBC suspension. Finally, ultra-pure cells were applied for the cell-SELEX procedure.

Aptamers are usually discovered and amplified by a sophisticated method named SELEX (abbreviations for Systematic Evolution of Ligands by Exponential enrichment) [28]. Through the SELEX procedure, a chemically synthesized library of random oligonucleotides (DNA or RNA or their modified forms; 40-100 nucleotides length, single-stranded) is exposed to the target molecules. Few oligonucleotides bind to the targets and Ligand-target complexes are then separated from unbound forms. Target-specific ligands are isolated and amplified for further processing. Initially, vigorously purified molecules were being used as aptamer targets; however, the SELEX procedure was gradually extended by the researcher to favor discovering aptamers against cells and complex matrices. The procedure in which cells are used as the target for aptamer binding was named cell-SELEX [9,29]. The cell-SELEX method introduced by Sefah was selected and followed for the current research.

Platelets, a disc-shaped anucleate cell in the blood, or thrombocytes are produced from bone marrow resident progenitor cells, named megakaryocytes, and 10^{11} cells per day are continuously being released into the circulation to maintain their normal count in the blood (150000-450000 cells per microliter) [30]. They are short-lived cells (lifespan: 7-10 days) with numerous fundamental functions, including, direct involvement in blood clotting, tissue repair/wound healing, angiogenesis, inflammation, cell proliferation, tumor progression, and metastasis [31]. Like other cells, the platelet cell membrane is made up of two layers of phospholipid and more than 50 classes of membrane-bound receptors mediate platelet interactions with the vessel wall, other cells, and soluble factors. Any congenital or acquired disorders of the receptors are associated with defects in platelet procoagulant activity and manifest as hemorrhagic diseases. A clear example of the disease is platelet-type von Willebrand disease, PT-VWD, an inherited platelet disorder caused by the enhanced affinity of membranous GPI-bα to soluble VWF [32]. Platelets are also actively involved in atherosclerotic cardiovascular disease. Atherosclerotic plaque rupture exposes damaged endothelium and facilitates platelet interaction with exposed vascular surface leading to platelet adhesion, activation, and thrombus formation. Platelet regulatory drugs (or anti-platelet drugs) are frequently administered in many clinical conditions and the drugs either antagonize platelet membrane receptors or inactivate key member(s) of the intracellular signaling pathways. For instance, aspirin inactivates the COX enzyme and clopidogrel antagonizes ADP receptor P2Y12 on platelets. These drugs have often a narrow therapeutic window with no antidotes if the intervention was associated with side effects. In addition, 'one dose for all strategy' was common with the use of antiplatelet agents, therefore, an increased risk of serious bleeding is always being expected with the interventions [33]. Moreover, increased resistance or enhanced susceptibility of patients to the drugs are common, while no criteria for predicting the situation exist [34]. Aptamer technology has facilitated the development of a new generation of therapeutic agents with target specificity and reversibility even after administration. For instance, Oney [35] and Nimjee [13], both developed RNA aptamers that bind VWF with high affinity and inhibit platelet adhesion and aggregation. Antidote molecules were also designed to quickly reverse aptamer function, a new important invention promising creation of safer and regulable drugs. Aptamers are oligonucleotides (DNA or RNA) composed of less than 80 nucleotides and bind with high affinity and specificity to the target molecules (ligands). Two or more aptamers can be combined and used for complex and unique purposes [36]. Aptamers immobilized on a solid surface can be used for the detection of target molecules [17].

Through the current study, we used a randomly

synthesized oligonucleotide library of single-stranded DNA molecules of 79 nucleotides. The sense primer was FITC labeled and antisense primer was phosphorylated in the 5' end. Polymerase chain reaction, PCR, was used for amplification of the strands, and lambda exonuclease was used for digestion of the phosphorylated strand. Purified human platelets were used as aptamer targets and non-specific oligos were absorbed to RBC and WBC from the same donor. Flow cytometry was used for the evaluation of the ligand enrichment process. The cell-SELEX procedure was progressed according to the Sefah [5] method and platelet-specific ligands were gradually enriched, which was demonstrated by increased fluorescence signals detected by flow cytometry. The frequency of the platelets stained with FITC-labeled aptamer reached the highest rate in the 11th cycle of the cell-SELEX and remained nearly unchanged during the 12^{th} and 13^{th} cycles. To the best of our knowledge, the production of platelet-specific aptamers has not been reported yet. The number of SELEX cycles required before cloning of the selected aptamers did not adhere to an especial rule. The number of cycles for developing aptamers against prion proteins by Bibby [37] was eight. Mehennaoui [18] operated 19 cycles of SELEX for producing aptamers against dexamethasone. Chen [38] used seven cycles of SELEX for producing aptamers against hepatic stellate cells. In our study, PCR products from the 12th round of SELEX were amplified using unlabeled primers and cloned. Positive colonies (200 colonies) were selected and propagated in liquid media. Plasmids were extracted and sequenced. Sequenced aptamers were aligned and seven representative aptamers were further evaluated for affinity determination and elucidation of the aptamers' effects on platelet function tests. The affinity of the aptamers to platelets ranged between 109 and 339 nM, which were comparable to the previous reports. The affinity of DNA aptamers discovered by other researchers is also in nanomolar levels. For instance, Affinity of DNA aptamers have been developed by Forier [14], Duan [39], Tang [40], Wu [16], Berg [41], Zhou [42], Spiga [43], Baig [44], Moon [45], Moosavian [46], Mozioglu [47] and Oney [35] were reported to be 1.2 nM, 32.24 nM, 669-998 nM, 23 nM, 137 nM, 4-12 nM, 200 nM, 210-1000 nM, 3.49 nM, 133-191 nM, 5.09 nM and less than 20 nM, respectively, (range:1.2-1000 nM) . Regarding the nature of target molecules on platelets, binding of all 7 aptamers to their targets was abolished after trypsin treatment of the cells, demonstrating the protein nature of the molecules. In addition, the pre-binding of three aptamers to their targets on platelets partially protected the targets from digestion by Trypsin. No one of the seven aptamers could change the results of the platelet function tests PFA-100 and LTA, considerably. The explanation for the failure is the high variability of the data obtained from the two tests. It seems that a new method with low variation and high reproducibility is needed for the evaluation of aptamer effects on platelet functions.

We isolated 200 colonies harboring aptamer-containing plasmids and evaluated seven representative aptamers targeting protein molecules on the platelets. Our results showed the discovery of platelet-specific aptamers through the cell-SELEX method was feasible and the affinity of the aptamers was in an acceptable range and favorable for application in research. Purification of the target molecules using aptamer-affinity column and further identification of the targets is our goal for a future study. These aptamers can also be used for the detection of human platelets in forensic medicine.

Materials & Methods

Oligonucleotides

HPLC purified, Single-stranded random oligonucleotide library consisted of 38 random nucleotides in the middle portion and two 21 and 20 constant sequences in the 5' and 3' ends, respectively (5'-GCCTGTTGTGAGCCTCCTAAC [N38] CATGCTTATTCTTGT-CTCCC-3') [48]. Pairs of primers (labeled/unlabeled) were ordered to "TAG Copenhagen, Denmark" (forward primer: 5'-GCCTGTTGTGAGCCTCCTAAC-3', reverse primer: 5'-GGGAGACAAGAATAAGCATG-3'). The forward primer was FITC-labeled and the reverse strand was 5'-phosphorylated. Unlabeled primers were used for cloning and sequencing experiments.

Buffers and antibodies

The "Dynabeads® M-280 Sheep anti-Mouse IgG" magnetic beads were from ThermoFisher (Cat. No:11202D). Anti-CD45, anti-CD-235a and anti-CD41 antibodies (ab8216/ab212432/ab11024 produced in mice) were from Abcam. Acid Citrate-Dextrose (ACD; C3821), Prostaglandin E1 (PGE1; P5515), Acetylsalicylic acid (ASA; A5376), and EDTA (ED2P) all were from Sigma-Aldrich. Annexin-V-FITC and anti-CD62p-PE were from Becton Dickinson (Cat. No. 556570 & 555524). Platelet washing buffer [49] was composed of NaCl (129 mM), KCl (2.8 mM), $NaHCO_3$ (8.9 mM), KH_2PO_4 (0.8 mM), $MgCl_2$ (0.8 mM), EGTA (2 mM), Glucose (5.6 mM), HEPES (10 mM), BSA (0.35%), pH:7.4, prepared in 1000 ml DW (final volume). The binding buffer was composed of the same ingredients except for EGTA that was replaced by $CaCl_2$ (1 mM). EGTA, as an anticoagulant, was included in the platelet washing buffer because it chelates Ca^{2+} ions with high affinity (compared to the EDTA) and prevents platelet activation. EGTA was replaced by $CaCl_2$ in a binding buffer because Ca^{2+} ions are necessary and facilitate interactions between oligomers and platelet-surface molecules.

Preparation of cells

Freshly prepared ultra-pure blood platelets were used as targets. Experimental conditions were optimized for preserving the intactness of the cells [24]. Blood was drawn using an 18-gauge needle through the intravenous cannula. Anticoagulant was made up of 18 ml ACD, 12 µl PGE1 (1mM), 120 µl ASA and 480

µl EDTA (0.5 M). Hematologic parameters were measured and platelet-rich plasma (PRP) was prepared by repeated centrifugation of the anticoagulated blood at 200 g.

Immunomagnetic separation

Magnetic Dynabeads were equipped with capture antibodies according to the manufacturer's recommendations. Anti-CD45 and anti-CD235a capture antibodies were used for WBC and RBC depletion of PRP preparation [50]. The magnetic field was used for precipitation of the bead-bound cells and floating cells (platelets) were separated and precipitated. Highly pure platelets were counted and resuspended in the washing buffer (Tyrode-Hepes buffer with EGTA). P-selectin (CD62p) is a platelet activation marker. Annexin-V binds to phosphatidylserine residue that is exposed on the surface of apoptotic cells. FITC-labeled Annexin-V and PE-labeled mouse anti-human CD62p antibodies were used for flow cytometric detection of the two markers in purified platelets [51].

Preparation of cells for counter selection

White and red blood cells (WBC/RBC) were prepared and used for counter selection. Anticoagulated blood was precipitated and a complete buffy coat section plus a small fraction of the underlying red cells were removed and resuspended in platelet-poor plasma (PPP) derived from the same blood sample. Magnetic Dynabeads equipped with anti-CD41 antibody were used for depletion of the platelets from the suspension. The viability of the WBCs was determined using Trypan blue exclusion method.

Cell-SELEX protocol

Cell-SELEX was performed obeying the procedure introduced by Sefah [5]. For folding into its 3D configuration, 5 nmol single-stranded oligonucleotide library was resolved in 1ml binding buffer, incubated at 95°C for 15 min, and cooled immediately. Ultra-pure platelets ($5x10^6$ cells) were washed 3 times with washing buffer and resuspended in 1 ml binding buffer. Both library and target cells were adjusted to room temperature (18°C) and mixed and then incubated for one hour at RT. Unbound oligos were separated by centrifugation at 200 g for 15 minutes followed by three additional washing steps. Bound oligos were eluted from the platelets by incubating diluted cells at 95°C for 30 min with gentle agitation followed by centrifugation at 800 g for 15 min. Eluted oligonucleotides from the first round of SELEX were purified and amplified through a preparatory PCR and single-stranded DNA was prepared by Lambda exonuclease and aliquoted as "control library".

PCR conditions

Eluted oligonucleotides (1 µl: 18pg), 10x PCR buffer (5 µl), primers (2 + 2 µl: 0.4 µM each), dNTP (2 µl: 0.4 mM) and $MgCl_2$ (0.5 µl: 0.5 mM) were mixed and then 0.3 µl (1.5 Unit) Taq DNA-polymerase was added. Finally, 37.5 µl DDW was added to reach the final volume (50 µl). PCR conditions were optimized and set to 5 min at 94 °C for initial denaturation, followed by 35 cycles of 60 sec at 94 °C for denaturation, 60 sec at 54 °C for annealing and 90 sec at 72 °C for extension and an additional 10 min at 72 °C was applied for final extension step. Agarose gel (3%) electrophoresis with TBE (0.5x) buffer as mobile phase and "DNA safe stain" (Sinaclon, Cat. No: EP5082) as the nucleic acid stain was used for visualization of the PCR products.

Single-strand preparation

PCR products from amplification of eluted oligonucleotides are double-strand, one strand is FITC-labeled and the other strand is 5' end phosphorylated. For application in the next round of SELEX, single-strand FITC-labeled oligonucleotides should be separated from complementary sequences in isolated form. Lambda exonuclease (ThermoScientific; #EN0561) is the unique enzyme that can break phosphorylated strands down while leaving the other strand intact. The reaction is simple and strand-specific and only digest phosphorylated strand. Reaction conditions were optimized and the reliability of the enzyme was confirmed through native polyacrylamide gel electrophoresis before application for the main study.

Desalting and extraction of oligonucleotides

PCR assay is susceptible to even a trace amount of impurities of salts in the DNA samples and desalting is a necessary step for successful amplification of the target sequence. Oligonucleotides (in the current study) are short DNA strands with 79 bp length and successful isolation of the short DNA sequences from samples requires the inclusion of some accessory materials, usually known as carriers. Linear polyacrylamide is a newly introduced carrier that is chemically inert and does not interfere with subsequent procedures. In addition, LPA can be produced with high purity in any laboratory setting. Isopropyl alcohol and ethyl alcohol are two DNA precipitating agents that show their advantages, while the required volume of isopropanol for DNA precipitation is half of the ethanol. There is no need for a cold incubation period (isopropanol, unlike ETOH, also works well at room temperature), however, ETOH works better for desalting. We, initially, concentrated DNA strands from diluted samples using isopropanol and then desalting was done using ETOH. LPA was used as a precipitation-assistant agent. For desalting and extraction, 200 µl of the eluted oligos were mixed with 20 µl sodium acetate (3M), 5 µl Linear polyacrylamide (LPA as a carrier; 2.5 µg/µl), and 170 µl cooled isopropyl alcohol. After centrifugation at 15000 g for 45 min, the precipitate was washed 2 additional times using 500 µl cooled ethanol and gently dried at ambient temperature, and reserved (or amplified to get ready for the next round). LPA (2.5 µg/µl) was prepared by the method of Gaillard [52].

Negative selection

Counter selection of eluted oligos was only done at the third round and beyond. Oligos eluted from the positive selection were exposed to platelet-free WBC/RBC cells suspended in binding buffer and unbound oligos were collected in the supernatant, amplified by PCR, single-stranded by exonuclease, desalted, purified, and aliquoted at -20 °C until the next round.

Monitoring the efficiency of the enrichment process

A total of 13 rounds of cell-SELEX were applied. That means; 13 times in a duplicate or triplicate experiments oligonucleotides from the initial library, or single-strand preparation from the previous round of SELEX, were exposed to target cells (platelets) then bound DNAs were eluted and exposed to non-target cells (RBCs and WBCs to remove non-specific oligos) and DNA from the final supernatant was extracted and PCR amplified. The stringency of the conditions, e.g., incubation time, elution time, duration of washing steps, and so on, was gradually increased to further enrichment of high-affinity platelet-specific oligonucleotides and efficient depletion of useless DNAs from the process. PCR products (double-strand) from each round of SELEX were incubated with Lambda exonuclease to single-strand FITC-labeled DNA preparation and used in the next round of SELEX. A total of 10 ssDNA samples from 4-13 rounds of SELEX were collected and used individually for flow cytometric detection of the efficiency of the enrichment process. Oligonucleotides originated from the first round of SELEX were hugely amplified and then single-stranded and used as control library (e.g., for drawing quadrans in flow cytometry diagrams. Briefly, $5x10^5$ freshly prepared and washed platelets were exposed to ssDNA samples in 100 µl binding buffer, incubated at 18 °C for 30 minutes in a dark place, and fluorescence signals for $5x10^4$ cells were acquired by the instrument.

Sequencing of the select aptamers

Selected ssDNA from the 12th round of cell-SELEX were amplified by PCR using non-labeled primers and Taq DNA Polymerase. Oligos from the 13th round of SELEX were omitted to prevent confronting with repeated emergence of identical colonies after cloning. PCR product was further purified using electrophoresis and eluted DNA was cloned to pTG19-T vector using a standard cloning kit (Vivantis; TA010). *E. coli* (TOP10) was used as a competent cell, and transformed cells were layered on LB-agar media enriched with ampicillin and IPTG X-GAL. White colonies were selected individually and reproduced in liquid media. Plasmids originating from a single white colony were extracted using alkaline lysis buffer and sequenced. Clustal Omega online multiple sequence alignment tool was used for alignment of the sequences. Configuration of the sequences was determined using RNA structure 6.2 software.

Affinity and specificity of the selected aptamers

Seven representative colonies were selected, reproduced individually and their plasmids were extracted. Seven aptamer sequences were amplified using FITC-labeled and phosphorylated primers. Phosphorylated strands were digested and FITC-labeled strands were further resolved by electrophoresis and extracted in the pure form. Seven FITC labeled aptamers (each in pure form) were considered for affinity determination experiments.

For affinity determination, in triplicate experiments, fixed numbers of target cells ($5x10^5$ platelets) were exposed, in binding buffer, to nine serial dilutions (0-1600 nM oligos) of FITC-labeled aptamers and incubated in a dark place at 18°C for one hour. After a washing step, fluorescence data related to $5x10^4$ cells were acquired by flow cytometry. Control library was used in parallel as a control. Each experiment was done using one aptamer, therefore, one-site saturation equation $Y=Bmax*X/(K_D + X)$ was used for the calculation of dissociation constant in which, Y is the mean fluorescence intensity, X is the aptamer concentration and Bmax is the maximum fluorescence intensity.

Specificity of the aptamers were tested using pure leukocytes, platelets and RBCs prepared from 5 donors with different characteristics regarding gender and age. Briefly, fixed number of the cells were incubated with fixed concentration of the FITC-labeled aptamers. Density of the cell-bound aptamers were assayed by flow cytometry.

Trypsin digestion of ligands

Exposure of platelets, $5x10^5$ cells, to trypsin (Sigma: T4549; 0.25% final concentration; 10 min incubation time; at 37 °C), before and after aptamer binding was used for evaluation of the ligand nature and accessibility. RPMI-1640 enriched with FBS (10%) was used for trypsin inactivation and centrifugal force was used for cell precipitation. One hundred µl containing 50 nm FITC-labeled aptamer was added to the cells and incubated at 18°C for 30 min. After washing, the cells were analyzed using flow cytometry. Control library and untreated cells were also included in the experiment.

In vitro effects of Aptamers on platelet function

We investigated by two conventional methods, Light Transmission Aggregometry (LTA) and platelet function analyzer (PFA-100), how aptamer binding to platelets can interfere with adhesion and aggregation of the cells. For LTA testing freshly prepared platelets (2×10^{11} cells/L in PPP) were exposed to three concentrations (0.01, 0.1, and 1 µM, final concentration) of the aptamers at 37 °C for 30 min. Platelet-aptamer complex (270 µl) was transferred to LTA cuvette and mixed with 30 µl agonist (type I collagen: 5 µg/ml or epinephrine: 10 µM) and agitated (800-1200 rpm). The aggregometer was calibrated using PPP (100% transmittance) and PRP (0%) and the light transmittance of the test and control tubes were measured steadily for 12 minutes. The slop and maximal aggregation rate (MAR) were calculated and compared. Any recognizable changes of platelet aggregation traces within pairs of test and control experiments were also noticed.

For PFA-100 testing, citrated blood was prepared and spiked with three different concentrations (0.01, 0.1, and 1 µM) of aptamers and incubated at 37 °C for 30 minutes before running the test. Both collagen-epinephrin (CEPI) and collagen-ADP (CADP) cartridges were tested.

Ethical considerations

All patients (volunteers) gave informed consent and signed an informed consent form. All samples obtained in this study were approved by the ethics committee of the "Research Ethics Committees of the Ferdowsi University of Mashhad" with the code of IR.UM.REC.1400.076 and the research was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects", (amended in October 2013).

Authors' Contributions

FA was fully responsible for study design, for conduction of all experimental procedures, and data collection and evaluation. Also, she was responsible for preparation of the manuscript and revising it if it was necessary. MS participated in the study design and supervision of the work. AH contributed in statistical analysis of the data and evaluating integrity of the data and interpretation of the results. GHT was responsible for overall supervision. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no conflicts of interest

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